THE HIGH TEMPERATURE ORGANISM OF FERMENTING TAN-BARK.

Part i.

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(Four Text-figures.)

In the manufacture of white lead, plates of metallic lead are generally corroded by the old Dutch fermentation process. As conducted at the works of Lewis Berger and Co. at Rhodes, near Sydney, the fermentation stacks are built up of layers of tan-bark with the lead contained in pots between successive layers of bark. A layer of bark some twenty-four inches deep is first laid on the floor, and on this are placed the earthenware pots each containing about three inches of dilute acetic acid and about eighteen perforated sheets of lead (buckles) all resting upon a ledge in the jar. The jars are covered with timber and on the timber a layer of sixteen inches of tempered tan-bark is placed; then comes a layer of jars, then timber, and so the stack is built up. Each tier of jars is connected with the air at the top of the stack by means of wooden chimneys, the vent of each chimney being covered with a piece of wood. When the fermentation is at its height, steam rushes out of the vents and the extraordinary thing is that the temperature of the issuing steam is in the neighbourhood of 180° F. This is a remarkably high temperature for there is some evidence in favour of the fermentation being due to the activity of micro-organisms.

The fermentation occupies about four months and, in its course, the lead is carbonated with an efficiency of from 60 to 80 %, while the temperature, starting at about 140° F., rises during the second month to from 180° to 190° and then falls to about 140° when the stacks are drawn. The bark darkens in colour, shrinks in volume and becomes less fibrous. It is used again, but in order to adjust the wastage and to improve the texture, it is mixed with one part of new bark for every four parts of old tempered bark when the stacks are made.

The tanners of Sydney use wattle-bark in preparing their tan liquors and Lewis Berger and Co. have found that the fermentation of this kind of bark requires a different treatment from the barks used in other parts of the world. A preliminary fermentation or tempering is necessary. This consists in storing the bark in a central reserve or alley and watering it daily for about a fortnight. The temperature of the mass rises to well over blood heat, but not to the temperature that it attains in the stacks. One can see lumps of bark on the top of

the heap covered with a white mould, and a portion taken from well inside the heap contained budding yeast-like cells. These were living, for they did not become stained by watery eosin. It is possible that this preliminary mixed fermentation at a comparatively low temperature brings about the production of certain nutrients which are necessary for the life of high temperature bacteria in the main fermentation. However this is a matter for future investigation.

A sample of bark was taken from a stack when the top tier was in active fermentation. Digging down about six inches, the temperature was found to be 180° F. and a sterile bottle was filled with the hot bark, taken to the laboratory and subjected to investigation.

It may be mentioned here that a sample of the tempered bark contained 50.5 % of water driven off at 100° and 51.7 % at 130° C. while the stack-bark lost 59.7 % at 100°, and 61.9 % at 130°.

The microscopic examination of a watery suspension of the bark showed stout rods of various lengths, and threads. It was thought that the latter might be actinomyces-forms but as an *Actinomyces* colony was only once found upon the plates, it is probable that they were bacterial threads. During the investigation, it was noted that the bacteria formed threads of various lengths very easily. They occurred in the older cultures and may be looked upon as degenerate or involution forms.

A high temperature organism was eventually obtained but before this occurred some observations were made which led up to the successful issue. As plate cultivation at 60° is not an easy method of isolating bacteria, an attempt was made at 37°. Unfortunately the colonies that developed would not grow at 60°. Had the incubation of the plates been prolonged, punctiform colonies would have appeared; one race of the thermophilic organisms was subsequently obtained in this way. A preliminary incubation of the bark with water was found to be necessary and it was shown, later, that an alkaline liquid was very much better. Colonies were obtained by stroking agar-slopes with suspensions of the incubated bark and also by smearing plates which in order to minimise the drying and condensation that occurs at 60° were wrapped in butter-paper and put into damp chambers or larger Petri-dishes. With regard to the tubes, the plugs were pushed down and the mouths loosely closed with ordinary wooden corks. Rubber caps perish quickly at 60°.

The first trials with ordinary nutrient agar were negative; no growths were obtained and this led to the preparation of a special saccharose agar medium. Later work, however, showed that the ordinary nutrient agar was quite good for growing the high temperature organism, and the negative preliminary tests must have been due to the ignorance of the necessity for a preliminary incubation of the bark with water or with dilute alkali.

The agar-medium suggested by the early test consisted of saccharose 1 %, peptone 0.3 %, meat-extract 0.2 %, potassium citrate 0.3 %, magnesium sulphate crystals 0.2 %, and calcium chloride 0.05 %, made neutral to phenolphthalein. It proved a very suitable medium but, as it had a tendency to soften and the slopes slid down in their tubes when incubated at 60°, the second batch was made with 2.5 % of agar. The first active growths were obtained on this medium from a portion of the stack-bark that had been covered with water and incubated at 60°. The suspension was smeared over an agar slope and in 20 hours at 60° a luxuriant growth had spread over the agar which was broken up with numerous

gas bubbles.* The mixed culture had decomposed the saccharose with evolution of gas. From this culture other slopes were smeared and a number of colonies were obtained. Although these grew well at 60°, they failed to produce gas. A more vigorous gas formation was obtained from the mixed culture derived from another flask of bark which had been covered with N/100 sodium hydrate and which itself showed signs of gas formation, although it was doubtful whether the bubbles entrapped in the bark were derived from the fermentation of the bark or from the air absorbed or caught in the spaces of the bark. The examination of the gassy agar-tubes, by tube and by plate culture at 60°, showed only one kind of colony and the organism was not a saccharose fermenter.

Although the fermentation of saccharose was not the object of the research, it seemed probable that a definite saccharose-fermenting bacterium would be more likely to be able to ferment cellulose than one which could not do so. Consequently a rather extended search was made for this active organism, and gradually it was determined that the gas fermentation of saccharose (and of dextrose) was the result of a condition and not due to an admixture with an unknown organism. During the search, notes were made upon the nature and activities of the thermophilic bacterium.

It is a stout rod of a general length of from 3 to 4.5μ but varying from 1.5 up to 15μ or longer. Its breadth is 0.7μ. Spores are formed terminally; they are oval at first, but become rounded. Their general size is 0.9 × 1.5μ. The rods when grown in saccharose media at 60° were Gram negative; at 37° they were Gram positive. Although non-motile in fluid media, the rods were studded with many peritrichous flagella.

The colonies on saccharose agar were white and rounded and often became irregular with age. They were either translucent white or semi-opaque and rough or terraced (button-like). Microscopically, they had generally a granular centre which gradually thinned off to a clear margin. Sometimes the structure appeared rippled or wavy and sometimes faintly radial. The edge was sometimes smooth, sometimes lacerate. Much seemed to depend upon the amount of spore formation that had taken place, and the consistency of the agar. An almost translucent colony with a wavy structure when transferred to sloped agar gave an opaque rough stroke and, conversely, an opaque colony gave a smooth translucent expansion.

Some races of the bacteria grew slowly at 37° while others did not. They all grew well at 60° and not quite so strongly at 65° or at 70°. At the latter temperature some races failed to grow, and this raised the suspicion that much had yet to be learned about the acclimatisation of the bacteria and their spores. Throughout the research there was always a doubt as to whether a particular culture would transfer. An actively growing culture would always transfer but the same could not be said of a culture which had been at laboratory temperature for days or weeks or at 60° for a few days.

When grown upon saccharose-agar coloured with litmus, the bacteria seemed to have a double action (see p. 83). With gradually increasing acidity, the

* This production of gas was an exceptional case, for later tests showed that a preliminary treatment in an alkaline liquid or the presence of alkali in the agar itself was necessary. But it must be borne in mind that when bacteria have been recently isolated from what may be called their natural habitat, they may be and probably are more vigorous than after a spell of subcultivation in the laboratory.

growths became depressed progressively, making it clear that an acid condition of the medium is unsuitable. In dextrose and saccharose litmus broths, the media became reddened and then bleached but the growth was feeble. Meat extract is not a suitable source of nitrogen. Urea and ammonium salts are quite unsuitable while on the other hand peptone and asparagin are good.

In peptone water with nitrate, the nitrate is reduced to nitrite.

In the primary isolation of the high temperature organism, growths were obtained from stack-bark which had been wetted with water and with N/100 sodium hydrate and, of the two, the soda contained the greater number of bacteria per loop of suspension. In following up this observation, stack-bark was put into bottles and wetted with dilute sodium hydrate of varying strengths. The liquids rose half way to the surface of the bark. The bottles were closed with corks fitted with glass tubes drawn out at one end to a capillary point in order to lessen the evaporation of the liquid contents. They were incubated at 70° and from these growths were obtained, but as no gas was produced in saccharose agar, it was considered that 70° was too high for laboratory work. Accordingly new portions of stack-bark were taken, wetted with sodium carbonate and incubated at 60°. The growth and gas-formation produced by stroking loops of the alkaline liquors on saccharose agar are noted in the table.

Bark with Na_2CO_3.	N/2·5		N/5		N/10		N/25	N/50
	growth	gas	growth	gas	growth	gas	growth	growth
1 day	+	O	+	O	+	O	O	O
4 days	+++	+	+++	+	+++	+	+	+
6 days	+++	O	+++	+	+++	+	+	+
10 days	+++	O	+++	+	+++	+	O	O
13 days	++	S	+++	S	+++	+	O	O
18 days	++	S	+++	S	+++	S	O	O

S=slight.

Another experiment with stronger dilutions of sodium carbonate was made and the following results were obtained.

Bark with Na_2CO_3.	N/0·5		N/1·25		N/2·5	
	growth	gas	growth	gas	growth	gas
1 day	+	O	++	O	++	+
5 days	O	O	+++	O	+++	+
8 days	O	O	++	O	+++	S

S = slight.

A bulk culture in N/5 sodium carbonate gave a gas-forming growth in two days, but not in one day.

The experiments show that N/5 or N/10 sodium carbonate is best for developing the organism that produces gas in saccharose-agar. The organism is contained in the condensed water of the slopes which show gas formation and when this water was seeded into litmus broth containing either saccharose or

dextrose there was a production of acid and of gas in 5 days at 60°.* The colonies that developed on the agar slopes contained the rod-shaped bacteria already noted, and as these did not produce gas, either in agar or in broth, it is evident that the gas-producer is difficult to obtain. Some seventy colonies had been picked out and none of them were gas producers. In films prepared from the condensed water, one could see the stout bacteria accompanied by long, thin, faintly staining rods. In old nutrient agar cultures, and especially in Hansen's fluid, thin rods with central granules or with terminal granules were noted and it therefore seems unlikely that the faintly staining rods are the gas-producing bacteria. By the negative method of staining one could see here and there structures which might be either spirochaetes or flagella but they were neither sufficiently numerous nor sufficiently decided to be the active agent. Although the large rod-shaped bacteria were non-motile yet when appropriately stained they were seen to have many peritrichous flagella and it is possible that a few were shown by the negative stain.

In dextrose broth containing various nitrogenous nutrients such as meat-extract, asparagin, urea or ammonium phosphate, acid and bleaching were produced in the first two and no gas was formed in any. The infecting material was the condensed water of a gassy culture, so that it is probable that the active organism does not grow freely in fluids.

A repetition of the experiment with the same kind of infecting material gave in the case of asparagin a production of gas by the 5th day. It was noted that by this time the growth was very considerable, the broth being quite opaque. It was also noted that on the day following, the gas-bubbles were absent and the volume of gas in the inner tube was less. The evolution of gas had apparently stopped on the fifth day and whether this was due to a lack of nutrition or to the sudden cooling of the culture on the fifth day during examination, further experiment must decide. The active liquid was, on the fifth day, seeded into new asparagin broth and one day later this was bleached, showing a turbidity at the surface. No gas developed even after twenty days.

It must not be considered that treatment of the bark is necessary to obtain a growth of thermophilic organisms. These can be obtained directly from the bark by rubbing fragments over an agar slope. These however do not produce gas in the medium.

Having apparently failed to obtain a pure culture of the active organism, it was decided to try the effect of an infusion of tan-bark. Accordingly a quantity of bark was mixed with twice its weight of water and heated at 60° for half an hour, strained and filtered. The infusion was quite gummy and had an acidity to phenolphthalein of + 0.85°. One c.c. of the infusion was added to saccharose-nutrient agar together with sodium carbonate to make the alkalinity = —24°. Upon the slopes colonies appeared and, at the same time, the medium was pierced with gas bubbles. Small colonies showed up on the second day and these were fished out and transferred to fresh slopes. The growths were all similar and apparently the same as had been obtained previously. No gas was formed on saccharose nutrient agar, but gas was produced in the same agar with the ad-

* An exceptional case. No gas was obtained in these media with the pure and necessarily older races. But it was the occurrence of this gas-production in the supposedly impure culture that prolonged the research until it became apparent that no other kind of organism could be obtained.

dition of the tan-bark infusion and sodium carbonate. The gas was probably not caused by the interaction of the tannin bodies with the sodium carbonate, for certain of the tubes which failed to show growth contained no gas bubbles. And as growth was necessary, it is possible that the faint acidity produced during growth may have given rise to the evolution of gas from the added carbonate. The gas bubbles disappeared when the tubes cooled down to laboratory temperature. It may be that the gum derived from the infusion assisted in holding the gas in the medium. Be this as it may, the colonies isolated from growths that gave gas formation, failed to produce gas in the absence of bark, and we may conclude that the gas in the bark-infusion tubes was due to the interaction of the infusion plus sodium carbonate and the acid formed by the bacteria from the sugar. An organism capable of fermenting saccharose directly was the object of the investigation at the moment.

A weaker infusion of tan-bark was made by adding three parts of water and letting the mixture stand at 22° for two hours. It was brighter and neutral and clearly contained very little tannin. Slopes of saccharose nutrient agar containing 11, 20, 27 and 33 % of this infusion were stroked with a pure culture but the growths obtained did not differ in any way from those without the infusion.

The strengthening of the saccharose nutrient agar by the addition of peptone, asparagin or meat-extract did not lead to the production of gas by the undoubtedly pure colonies. Thus up to this point the isolation of a saccharose fermenter in pure culture had not been accomplished.

About this time it was noted that the stack-bark, originally covered with dilute sodium carbonate* had a slight covering of a white mould. This consisted of a mass of aerial hyphae or conidiophores sprouting from the surface of the bark and carrying sessile or nearly sessile conidia along the length of the thread. They were quite short, about 0.14 mm. in length and the conidia seemed to be double and 10μ long when measured in air with a Leitz No. 8 objective. When immersed in water, the conidiophore measured 1.4μ in diameter and the conidia were oval or spindle-shaped, the latter shape being caused by a collar at one end, probably the remains of a short pedicel. In size they ranged from 2.8-3.5 × 4-6μ. The length proved that when noted growing in the air, the conidia were in couples. The contents were granular and one rather long conidium in a stained film showed a light central portion suggestive of the possibility of the cell being able to divide in two. The mycelia in stained films showed as unstained threads with irregularly placed, deeply stained granules and very similar to the threads noted in the films prepared from the condensed water of active mixed bacterial cultures.

Attempts were made to pick off the minute conidia from the bark and sow them upon solid media for incubation at 60° but the results were disappointing; either a strong growth of the inactive thermophilic rod was obtained or the tubes remained sterile. Hanging drop cultivation was also without result. Increasing the acidity and the alkalinity of the media, using the condensed water of an active growth as the infecting material, were useless for augmenting the

* The stack-bark had been covered with N/5 sodium carbonate and kept at 60° for two days when the fluid was found to contain active gas formers. On the fourth day the liquid was used in testing for filter passers and the residual bark was half covered with N/10 soda. Four days later the liquid contained gas formers, and in another four days the mould was noted covering many fragments of bark.

growth of the active gas producer. The familiar spore-bearing rods persisted in coming up and no other organism could be isolated.

A bottle of active stack-bark originally covered with N/5 soda had been set aside in the incubator at 37° and had grown a good crop of *Aspergillus*. The same mould appeared in the original sample of stack-bark and had apparently survived the action of the soda and the lengthy exposure at 60°. With the idea that possibly this might be the active agent, a tube of saccharose nutrient agar was infected with the spores and incubated at 37°. In three days, gas bubbles appeared in the medium under a dense mycelial growth. It was also sown in combination with the thermophilic rod and incubated at 60° but there was no growth of mould apparent and no gas formed. An attempt to acclimatise the *Aspergillus* to 60° by gradually raising the temperature from 37° was a failure and the conclusion was come to that the *Aspergillus* was not responsible for the fermentation of the saccharose.

It had been noted that the fluid taken directly from an alkaline bark liquor and sown on saccharose nutrient agar, gave a good growth of bacteria and a medium blown up with gas bubbles. The turbid condensed water at the base of the slope, when transferred to a second agar-slope, sometimes gave rise to gas production but generally did not. Colonies picked from the first tube never produced gas. Thus the gas production was fugitive.

The failure to obtain a gas-forming organism in pure culture and indeed to obtain any organism capable of growing at 60° other than the drumstick rod led to the idea that probably an alkaline condition of the agar was necessary for the evolution of gas, just as it was necessary to stimulate the growth of the cells from the bark. Possibly enough alkali was contained in the large loop used for seeding the tubes from the alkaline bark liquids, and as this disappeared on subculture so did the gas formation.

The saccharose nutrient agar had an acidity to phenolphthalein of + 2.2° and tubes of this were treated with increasing quantities of normal sodium carbonate and seeded with the condensed water of an active culture. Growth and gas were obtained on media having an alkalinity of — 14.4° and over, but not in media ranging from + 2.2° to — 12°. The limiting amount for growth appeared to be about — 24°, for with this alkalinity and with — 26.3° the agar had to be seeded several times.

Some of the races which had been isolated at different times, and were presumably pure, were grown on agar brought to approximately — 13° with Na_2CO_3. Out of 16 races, two failed to grow, three gave a growth of cells and no gas while the remainder produced growth with gas. The inactive bacteria could not be distinguished from the others.

The gas seems to be produced from the sugar, for tubes of alkalised nutrient agar without sugar gave luxuriant growths without gas when seeded with a culture which gave gas in the presence of sugar.

Thus we arrive at the fact that the drum-stick rod is the gas producer, and that the production of gas from saccharose by the pure races of the bacterium depends upon an alkaline condition of the medium.

An experiment was made with saccharose nutrient agar coloured with litmus and treated with increasing quantities of sodium carbonate. The slopes were stroked with an active race.

Alkalinity.	Notes.
+2° −1·7° −5·4° −9·1°	(one day): all pale red below condensed water level, condensed water strongly red, top of slope quite blue, gas bubbles in agar of −9·1°. (two days): showed progressive bleaching from +2° to −9·1°, upper parts of slopes quite blue, lower parts reddish, gas bubbles have disappeared from agar of −9·1°. (three days): +2° and −1·7° purple at top of slope, −5·4° and −9·1° blue at top of slope and body of agar bleached.
−12·8° −16·5°	(one day): no growth, reseeded with −9·1° culture. (two days): good growth, colour bleached below condensed water level and agar cracked with gas bubbles ; lower 2 cm. of slope red and upper 3 cm. quite blue. (three days): gas bubbles in both, −12·8° blue and −16·5° purple at top of slope.

From these notes it is evident that the bacteria produce acid from saccharose, that the gas production begins when the alkalinity is about — 9.1° and is pronounced at about — 12.8° and that the acid is possibly volatile at 60°, as was shown by the rather persistent blue colour of the thin layers of medium.

It had been found that the ordinary fluid media of the laboratory were not well suited for the growth of the bacterium and that urea, meat extract, and ammonia were useless as sources of nitrogen. Peptone water gave a fair growth, while asparagin was a good nutrient. To see how far an alkaline condition would improve matters, an experiment was made with meat extract, ammonium phosphate, urea and asparagin all 1 %, with 1 % dextrose, salts and litmus solution. Each tube received an increasing amount of sodium carbonate.

Absolutely no growth occurred with urea and ammonia. Meat extract developed an acidity with the control and — 4°, and no growth appeared in the others.

In from two to six days the asparagin became acid and bleached with 0, — 4°, — 8° and — 16°, but no growth occurred in — 20°. In 16 days gas had shown in the tubes with from — 4° to — 12°, and, by the 20th day, gas had developed in the control. Fresh tubes of asparagin were seeded from the — 8° culture and these showed acid and bleaching in three days with the same strengths of alkali as before. On the eighth day all the tubes up to — 16° were bleached and contained gas. The fluids were all alkaline to litmus paper. There was no growth in — 20°.

Litmus milk in five days was digested, bleached and was alkaline to litmus paper. Unsterilised milk in four days was partly digested and was alkaline to litmus paper. Several controls were unaffected.

Meanwhile experiments were in progress to see if the thermophilic rod could ferment tan-bark. The bark after sterilisation at 150° for an hour, was wetted, seeded with the organism and heated at 60° in a current of air. Several experiments which could only be considered as preliminary showed that certain precautions were necessary. Heating the bark in flasks in an incubator at 60° was unsuitable, for the hot dry air caused the rubber corks to harden and the rubber connections to loosen, with the result that the air and carbon dioxide were

sucked into the apparatus. Two flasks in series, each containing 50 c.c. of N/10 baryta water, coloured with phenolphthalein, were found to suffice for trapping all the CO_2. The decolorisation of the first flask was the signal for titrating the liquids in both flasks. The flasks themselves were fitted with wide tubes to avoid any tendency to blocking by the barium carbonate, which is largely deposited around the end of the inlet tube. It was unnecessary to have a flask of water in the thermostat connected with the other containing the bark in order to prevent the latter becoming too dry.

In the experiment about to be described the air passed through a tube of soda-lime, then through a Woulff bottle containing N/3 baryta water, thence through the flask with the bark, through two Wanklyn flasks in series containing baryta water and sometimes through an air-regulating flask to an air-reservoir connected with a suction pump.

The stack-bark that was used was acid, and a rough test indicated that the acidity was about 2.25 c.c. of N/1 acid per 100 grams of dry bark. The acidity is of some importance, because we have seen that the thermophilic bacteria develop from the bark only when it is made alkaline. The gas in saccharose media also formed in the presence of a certain amount of alkali. One might think, therefore, that in the stack, the bark would make the attached water acid, and thus prevent the growth of the bacteria and the fermentation of the bark. But conditions that affect bacteria in the laboratory do not have the same influence on the large or manufacturing scale. The acetic bacteria, for example, work at a temperature of 45° in a 5,000 gallon vat and they would not grow at this temperature in the laboratory in small bottles. The thermophilic rod from the stack-bark grows at 80° in the corroding stacks, but it grows best at 60° in the laboratory. It must also be remembered that in the laboratory we desire to see results in a few days while on the large scale, as in lead corrosion, the fermentation goes on for several months. The slow fermentation will probably ensure a better corrosion and in any case a rapid evolution of gas, if it could occur, might be of the nature of an explosion. So much for the condition of temperature. With regard to the acidity, one can imagine that bacteria will slowly produce change in large masses of fermentable organic matter such as silage, or acetic wort having an acidity that would render them inactive on a laboratory scale.

Some of the tempered bark was dried at 130° and divided into two portions of 30 grams each. These were heated in the oven at 150° for two hours. Each was treated with 20 c.c. of N/10 sodium hydrate and 50 c.c. of water, but in the case of the test flask the water contained a suspension of bacteria (race 73). The flasks were connected up with the apparatus previously described.

Evolution of Carbon Dioxide from Tempered Bark.

	Control. Milligrams of CO_2.		Test. Milligrams of CO_2.	
Days		Total		Total
1	56	56	107	107
2	73	129	36	143
4	54	118	41	184
5	19	202	31	215
6	31	233	43	258

Films prepared at the end of two days showed deeply staining rods in each flask, and tubes seeded with the liquors on the fourth day, gave bacterial growths both in test and control. It is clear that an exposure to 150° for two hours was not sufficient to sterilise the bark and thus the production of the same amount of carbon dioxide in four days is explained. It was thought that the speed of the current of air passing through the flasks would have an influence upon the results, but later observations showed that the quantity passing through was sufficient to sweep out all the CO_2 produced. The quantity passing through per hour varied from 1.3 to 2.1 litres, with an average of 1.75.

Pending the arrival of a quantity of stack and tempered barks, use was made of tan-bark in an experiment similar to the preceding. The preliminary experiments did not promise that a satisfactory result would be obtained from the untempered tan-bark, and it may be that the tannin remaining in the bark restricts the activity of the bacteria. The bark was dried at 130° and two portions of 24 grams were weighed into flasks and sterilised at 150° to 190° for three hours. This treatment was very drastic, but the previous experiment had shown that two hours at 150° was not enough to destroy all the spores. The control bark was wetted with 40 c.c. of N/20 sodium hydrate and the test with the same number of c.c. containing a suspension of the rods and spores of race 552 which actively decomposed saccharose in alkaline agar.

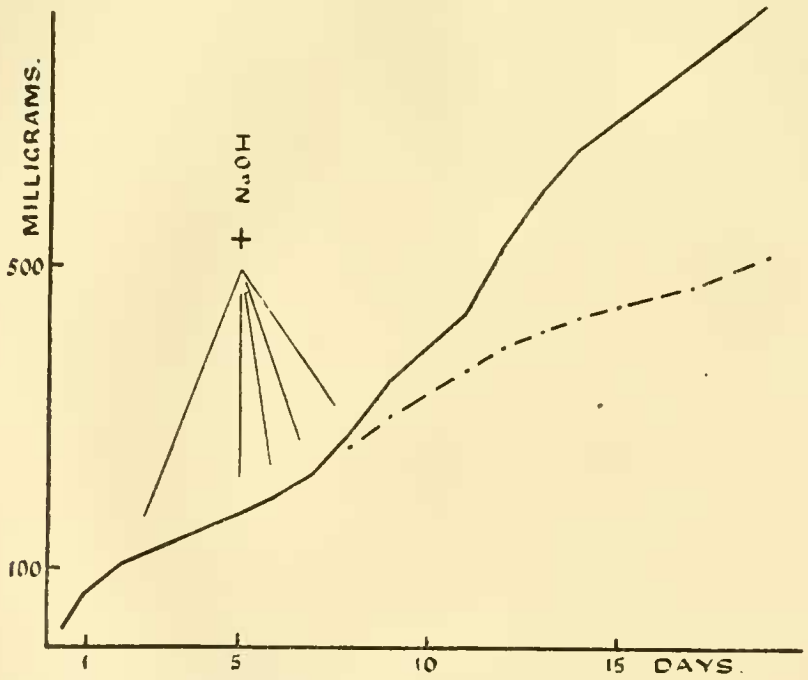
Evolution of Carbon Dioxide from Tan-bark.

Days.	Control. Milligrams of CO_2.		Test. Milligrams of CO_2.	
		Total.		Total.
	+ 40 c.c. N/20 NaOH			
1	73	73	72	72
2	40	113	37	109
	+ 10 c.c. N/10 NaOH			
5	61	174	69	178
	+ 20 c.c. N/10 NaOH			
6	24	198	24	202
	+ 5 c.c. N/1.85 NaOH			
7	25	223	29	231
	+ 10 c.c. N/1.85 NaOH			
8	44	267	55	286
	+ 10 c.c. N/1.85 NaOH			
9	41	308	63	349
11	60	386	93	442
12	26	394	86	528
13	20	414	70	598
14	19	433	56	654
15	15	448	41	695
17	29	477	74	769
19	37	514	84	853

Films were prepared at the end of the first day, and these showed faintly staining rods and spores in the test fluid and nothing in the control. At the end of the second day the control fluid was sterile and the test contained living bacteria. As the liquid was found to be acid to litmus, 10 c.c. of sodium hydrate were added to each flask. Upon the succeeding days, the acid reaction

of the fluids led to further additions of alkali, as noted in the table. It was observed that the bark became darker in colour as time went on.

At the end of the fifth day, the difference in the results was very small and the conclusion was come to that the raw bark was useless for showing the activity of the bacteria. In order to utilise the apparatus pending the arrival of a quantity of tempered bark, the experiment was continued and it is fortunate that it was, for the later results showed that under the conditions there was a small but decided evolution of CO_2 from the bark impregnated with the bacteria. The repeated additions of alkali gave more liquid than was desired, but this could not be avoided. Were the experiment to be repeated, the weak alkali would naturally not be used. At the end of the eighth day, the test contained living bacteria and the control was sterile.



Text-fig. 1. The Fermentation of Tan-bark. Total yield of Carbon Dioxide. Unbroken line = test, broken line = control.

From the experimental results and from the curves, one can see that the bacteria were quiescent for six days and, from that time onwards, they began to attack some constituent of the bark and from it to produce carbon dioxide.

Thus we have a production of carbon dioxide due to what we may call a chemical oxidation, as evidenced by the control test, and also to a bacterial fermentation, as shown by the excess of the test over the control. On the tenth day, the fluids in the flasks had a slight acidity towards litmus paper. Although it is possible that the repeated addition of the alkali favoured the growth of the bacteria, yet an examination of the curves gives one the idea that it was re-

sponsible for the concave depression of these curves and that it may not have had any decided influence in the production of the carbon dioxide.

It seems that this bacterium is one that has to get accustomed to its environment, for it was slow to produce carbon dioxide from the tan bark, and it is slow to grow on alkaline media as shown in experiments with increasing alkali where the slopes with a comparatively high alkaline content had to be repeatedly seeded from growths on agar with a little less alkali. It may be that in the tan-bark there is a constituent, e.g., tannin, which prevents the ready growth of the bacteria.

The addition of sodium hydrate to the flasks containing the bark was suggested by the increased activity of the bacteria in alkaline solution. But there was another reason for its use. On a former occasion (These Proceedings, 1918, p. 162), I showed that the heating of leaf-mould for two hours at 130° resulted in the formation of acid, the barium salt of which was largely soluble in water. The amount produced was equivalent to about 31 c.c. of normal acid per 100 grams of dry organic matter. It is probable that a higher temperature will produce a larger amount of acid and that a bark after sterilisation in the laboratory will be more acid than it was before sterilisation. Thus the addition of alkali to neutralise this developed acidity was indicated.

An attempt to arrive at the amount of acid developed during sterilisation was made upon a sample of air-dried tempered bark. Ten gram portions were weighed out, one was treated with 100 c.c. of water, another was sterilised at 200° to 176° for two hours, and then treated with 100 c.c. of water. After three days, the liquids were filtered and diluted one half. Using Sorensen's fifteenth-normal solutions of primary and secondary phosphates and brom-cresol-purple as the indicator, the extract of the air-dried bark had a P_H. number of 6.8 and the sterilised bark of 6.47. Thus an increase in the acidity of the bark following the sterilisation is shown. As the extract of the sterilised bark was brought up to P_H. 6.8 by the addition of 0.1 c.c. of N/100 alkali per 5 c.c., it appears that 100 grams of air-dried bark during sterilisation developed an amount of ionic acidity equal to 4 c.c. of tenth normal acid. This is very much less than had been expected from the experiment with leaf-mould, and seems to show that far too much alkali had been added to the tan bark in the fermentation experiment. Still the alkali had been added on account of the liquor reddening litmus paper.

A fermentation test was made with tempered bark, but it proved a failure. The bark was air-dried, then dried at 130°, and of this dry bark, 40 gram portions were put into flasks and sterilised at 150° for half an hour, and at 170° for an hour and a half. The flasks received 70 c.c. of water, one containing a suspension of the bacillus. By the second day, the seeded flask had given off 206 milligrams of CO_2 and the control 121 milligrams. The fluids in both flasks were at this stage shown to contain living bacteria and it followed that the tempered bark had contained some very resistant spores. Cultures from the control flask showed the rod with terminal spores growing at first as translucent colonies and rather gummy. By the time that the impurity of the control had been demonstrated, the amount of CO_2 given off was 371 milligrams in the seeded flask and 329 in the control. Five grams of powdered copper sulphate were added to the control flask and the fermentation was continued. By the seventh day the seeded flask had given off 597 and the control 553 milligrams of CO_2, and the control still contained living bacteria. The copper sulphate had been added as a disinfectant, but it would seem that the constituents of the bark had

reacted with the salt, annulling its disinfecting action. The constant increase of the seeded over the control flask was probably due to the initial activity of the introduced organisms.

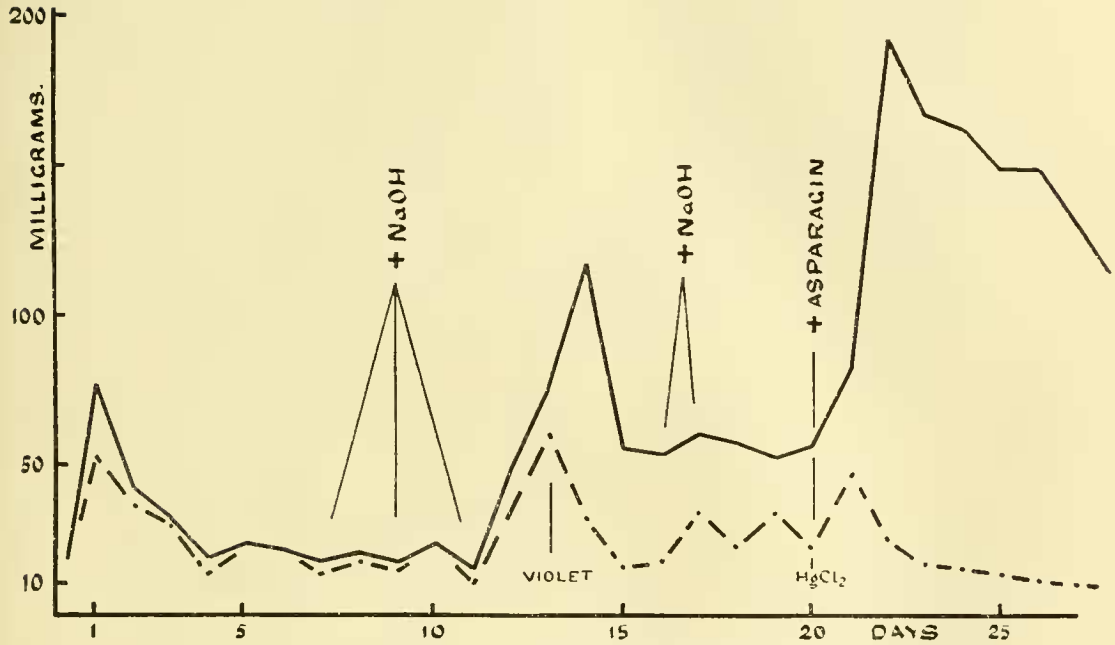
A fermentation experiment was made with tan-bark. Thirty grams of the bark dried at 130° were heated until a thermometer inserted in the control flask rose to 200°, then the temperature was slowly reduced to 164° during three-quarters of an hour and it was kept at this temperature for an hour and a quarter. Altogether the bark was sterilised for two hours at from 164° to 200°. The test flask was treated with 50 c.c. of water containing a suspension of race 80 which had been obtained from the control heated bark of the previous experiment.

Evolution of Carbon Dioxide from Tan-bark.

	Control. Milligrams of CO_2.		Test. Milligrams of CO_2.	
Days.	Daily	Total	Daily	Total.
1	53	53	77	77
2	36	89	42	119
3	29	118	32	151
4	13	131	18	169
5	22	153	23	192
6	21	174	21	213
7	13	187	17	230.
10 c.c. N/10 NaOH added.				
8	17	204	20	250
9	14	218	17	267
10 c.c. N/1.85 NaOH added.				
10	22	240	23	290
11	10	250	14	304
10 c.c. N/1 NaOH added.				
12	35	285	47	351
13	60	345	75	426
14	31	376	117	543
15	15	391	55	598
16	17	408	53	651
17	34	442	60	711
10 c.c. N/1 NaOH added.				
18	22	464	57	768
19	34	498	52	820
20	22	520	56	876
0.25 grams asparagin added.				
21	47	567	83	959
22	24	591	192	1151
23	16	607	167	1318
24	15	622	162	1480
25	13	635	149	1629
26	11	646	149	1778
27	10	656	131	1909
28	9	665	113	2022

On the seventh day the nature of the curve of the daily yields showed that a condition of equilibrium had been reached and something was needed to accelerate the evolution of gas. A previous experiment with the same bark had shown that alkali acted as such, and accordingly 10 c.c. of alkali were added to test

and control. The quantity advisable to add was as yet unknown and tenth normal sodium hydrate was used tentatively. This did not seem to improve matters and after the ninth day 10 c.c.* of N/1.85 sodium hydrate were added as a stronger alkali was apparently needed. The quantity of alkali added to each flask was capable of fixing 119 milligrams of CO_2, and as the yield was not completely depressed it is clear that the alkali was taken up by the bark and that it can absorb or fix much more alkali than the study of the aqueous extract would lead us to expect (see p. 87).



Text-fig 2. The Fermentation of Tan-bark.—Daily Yields of Carbon Dioxide. Unbroken line = test, broken line = control.

A slight increase on the tenth day followed the addition of the alkali, but on the eleventh day the yield went down. This suggested that the amount of alkali had not been enough, and accordingly 10 c.c. of N/1 sodium hydrate were added to each flask. This quantity seemed to be about right for the yield of gas began to rise immediately. Thus the thirty grams of dry bark required an amount of alkali equivalent to 16.4 c.c. of normal sodium hydrate to neutralise the inherent acidity or, at any rate, that part of the acidity which hindered the fermentative activity of the bacteria. This is equivalent to 54.7 c.c. of normal alkali per 100 grams of dry bark.

The control remained sterile until the last addition of soda. Apparently the spores had not been destroyed by the heat sterilisation and had remained quiescent until conditions of growth were made favourable by the alkali. The vegetating bacteria added to the test at the start remained in the acid bark for some time, but they slowly disappeared for on the twelfth day they were very few in number

* This contained 0.22 milligrams of CO_2 as impurity.

compared with earlier examinations. So pronounced was this that the flask was reseeded on the thirteenth day, with the same race, 80.

When the bacteria appeared in the control, 0.5 gram of crystal violet was added and this caused a sharp fall in the evolution of gas. The activity of the bacteria had been checked and the carbon dioxide that appeared on the fourteenth day may be looked upon as partly residual and partly chemical. The disinfectant did not destroy all the bacteria, but it seemed to hold them in check. On the nineteenth day living bacteria were still in the control flask and on the twentieth day 0.5 gram of mercuric chloride was added.

The rise on the fourteenth day was followed by a fall on the fifteenth and as the yield remained steady for another day, it was considered that a further addition of sodium hydrate might be made. Five c.c. of normal soda were added to each flask and although this caused a slightly increased evolution of gas yet the increase was so little that a further 10 c.c. of soda were added. The yields remained almost constant for several days, and the conclusion was made that the amount of alkali added up to the eleventh day, viz., the equivalent of 16.4 c.c. of normal sodium hydrate was sufficient for the 30 grams of bark. On the twentieth day it was considered that the experiment had reached an end and, as a final cast, it was decided to try the influence of the addition of a nitrogenous nutrient. The previous experimental evidence was in favour of asparagin, and accordingly 0.25 gram was added to each flask.

The effect of the asparagin was very marked and clearly indicated the necessity of the addition of a nitrogenous nutrient for a very active fermentation of tan-bark. There is, of course, the possibility that the carbon of the asparagin was quickly oxidised to carbon dioxide and the increase was derived from the asparagin directly. If this were the case, the 0.25 gram of asparagin is capable of giving 333 milligrams of CO_2. Before the addition of the asparagin, the evolution of CO_2 had been fairly constant at 55 milligrams. During the eight days following the addition, the excess over the 55 milligram mark totalled 623 milligrams, which is more than could be credited to the asparagin. It follows that there is an insufficiency of nitrogen in the bark for its complete fermentation.

An experiment with tempered bark, and parallel to the last with tan-bark, was started one day later. During sterilisation, the temperature, starting at 164°, rose in an hour to 200°, then fell gradually to 176° by the end of the second hour. Thirty grams of the bark dried at 130° were taken for each portion. Fifty c.c. of water containing a suspension of race 80 were added to the test flask and the same quantity of water to the control.

Evolution of Carbon Dioxide from Tempered Bark.

Days.	Control. Milligrams of CO_2.		Test. Milligrams of CO_2.	
	Daily	Total	Daily	Total
1	156	156	180	180
2	104	260	125	305
3	90	350	103	408
4	107	457	104	512
5	71	528	70	582
6	56	584	65	647
10 c.c. N/10 NaOH added.				
7	45	629	63	710
8	44	673	53	763
10 c.c. N/1.85 NaOH added.				
9	38	711	41	804
10	37	748	37	841
10 c.c. N/1 NaOH added.				
11	35	783	51	892
12	45	828	58	950
13	35	863	65	1015
5 c.c. N/1 NaOH added.				
14	26	889	58	1073
15	29	918	41	1114
16	35	953	35	1149
10 c.c. 5N/1 H_2SO_4 added.				
17	24	977	27	1176

The behaviour of this experiment was very much the same as the preceding; it was started a day later. There was a greater evolution of gas, both in the test and the control, which showed that tempered bark is undoubtedly better than raw tan-bark for the production of carbon dioxide. On the tenth day bacteria were found in the control and 0.3 gram of iodine was added on the eleventh day, but this did not destroy the bacteria, for slopes smeared on the thirteenth day grew a good crop of cells. As crystal-violet had checked the bacteria in the tan-bark control, 0.5 gram was added to this control. The addition of the 10 c.c. of N/1 sodium hydrate did not have the same effect as with the tan-bark from which it would seem that either too much or too little had been added. As the latter seemed more likely, 5 c.c. of N/1 sodium hydrate was added to each flask. This caused a fall and, as there seemed to be no likelihood of further information being obtained by continuing the experiment, 10 c.c. of dilute sulphuric acid (= 5 N/1) were run into each flask. The small yield of CO_2 following this treatment showed that all the alkali previously added had not fixed any appreciable amount of carbon dioxide.

On the whole the experiment was far from satisfactory on account of the non-sterility of the bark and especially of the control. The earlier high yields from the control, which were considered to result from a chemical oxidation, were, in view of a later experiment, the product of the activity of bacteria entrapped in the pores of the bark fragments. The sterilisation of the bark is an exceedingly difficult matter.

The loose water in the control flask was found to be sterile when tested on the second and fourth days, but the presence of the bacteria on the tenth day, when taken in conjunction with the amount of carbon dioxide evolved during

the first few days, suggests that the bacteria were actively fermenting while held in the pores of the bark. A later experiment with tempered disinfected bark yielded 82 milligrams of CO_2 in three days as against 250 in this experiment.

The addition of sodium hydrate to neutralise the inhibiting acidity of the bark seemed to be faulty: there was too much of the hit or miss method about it and one would like to get a more definite process. Some years ago, I showed that the organic matter of rotted leaf-mould could absorb alkali from solutions and there was a difference in its action upon the bicarbonates of the earths and the hydrates. Calculating upon 100 grams of dry ash-free leaf-mould, it was found that about 100 c.c. of normal alkali were taken up from the bicarbonates and about 450 c.c. from the hydrates. Thus there were two kinds of acidity. If there are two kinds of acidity in the tan-bark, it is probable that it is the kind which can decompose the bicarbonates that inhibits the growth of the bacteria.

More definite information regarding the base-absorbing power of the barks was obtained by placing two grams of dry bark in a bottle with 150 c.c. of N/10 baryta water and testing the loss from day to day. A similar test was made with magnesium bicarbonate. The numbers that follow are the c.c. of normal alkali absorbed by 100 grams of the dry bark.*

Baryta absorbed:—

Days	1	2	3	4	5	6
Alley-bark	433	457	474	476	485	491
Tan-bark	391	415	426	433	440	448

Magnesium bicarbonate absorbed:—

Days	1	2	5
Alley-bark	135	136	138
Tan-bark	52.5	70	73.5

The amount of alkali absorbed by the tan-bark from the bicarbonate in one day is close to that which was added in the fermentation test by the twelfth day, viz., 16.4 c.c. for 30 grams, which is equivalent to 54.7 c.c. of N/1 for 100 grams. This seems to be the optimum quantity required for a good fermentation because, when more was added on the sixteenth and seventeenth days, there was practically no further increase in the production.

In the first fermentation experiment with tan-bark (p. 85) the alkali added was equivalent to 18.5 c.c. N/1 for 24 grams of bark, i.e., 77 c.c. for 100 grams.

A second fermentation experiment was made with alley-bark. In the first experiment the alley-bark had been heated to from 164° to 200° for two hours and this was not sufficient to sterilise it. In this experiment, it was heated to 176° and in half an hour the temperature rose to 205°, from which it slowly fell to 186° in two hours; it remained at this for another hour. In order to make sure that the control at least would be sterile, 50 c.c. of 1 % mercuric chloride in 1 % sodium chloride were added and 50 c.c. of water were put into the test flask. Each flask contained 30 grams of alley-bark dried at 130°. As the barks did not wet readily, the flasks were steamed for an hour on the following morning and subsequently remained at laboratory temperature (26°) for five days when space was available in the thermostat. The flasks were connected up and kept at 60° until any mechanically fixed CO_2 might be eliminated before the test flask was seeded with bacteria. The difference in the

* The dry tan bark contained 4.42 % and the alley bark 11.74 % of ash.

amounts of CO_2 evolved, however, indicated that the test bark was probably not sterile, and an examination on the second day showed that this was the case. The gas-forming rod with terminal spore was found, not in the loose water, of which there was very little, possibly 2 c.c., but in the fragments of bark and it was only in contact with these that growth occurred on the agar slopes. The control bark was sterile. There was no need to seed the test flask for it already contained the active organisms.

The amount of CO_2 given off from the test was considerable and this without the addition of alkali or other substance. The small amount given off from the control, showed that what had previously been considered to be a chemical production of CO_2 was in all probability largely due to the activity of the bacteria in the supposedly sterile but actually non-sterile bark.

After the gas from the test flask reached the peak on the third day, the yield slowly fell and, on the sixth day, it was considered that the experiment had shown all it could under the conditions.

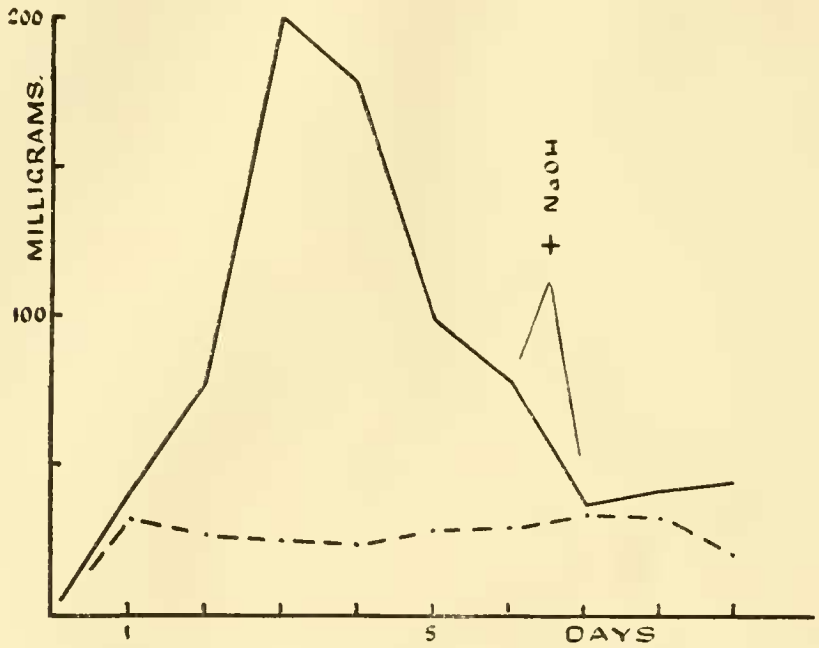
In the first experiment with the alley-bark, the addition of sodium hydrate had led to no increase in the evolution of CO_2, a circumstance which was in marked contrast with the tan-bark. The barks undoubtedly look different, and the alley-bark is more of the nature of charcoal, being black and brittle. Chemically the alley-bark absorbs more alkali from the earthy bases and bicarbonates than tan-bark and, if the fermentation of the latter is influenced by the addition of alkali, the alley-bark should also be favourably assisted. The inactivity of the alkali in the first experiment was peculiar, but possibly there had not been enough added. The tan-bark was favourably influenced by three-quarters of the alkali capable of being absorbed from bicarbonate in five days. In the same ratio, 30 grams of alley-bark should be influenced by 30 c.c. of normal alkali and it may be that the amount added in the first experiment, viz., 16.4 c.c., was not enough to produce any effect. But as 30 c.c. seemed a large quantity to add, it was decided to add 25 c.c. of N/1 sodium hydrate to each flask and see what happened.

Nothing did happen; the yield continued to fall. Next day a further 5 c.c. of sodium hydrate was added, making the total alkali in each flask 30 c.c. Two days afterwards the experiment was concluded as no increase in the CO_2 production had ensued. We must conclude that alley-bark is not influenced by the addition of sodium hydrate and differs from tan-bark in this respect. The control on the 7th day was sterile, while the test was replete with living bacteria.

Evolution of Carbon Dioxide form Alley-bark.

Days.	Control. Milligrams of CO_2.		Test. Milligrams of CO_2.	
	Daily yield.	Total.	Daily yield.	Total.
1	32	32	41	41
2	26	58	77	118
3	24	82	199	317
4	23	105	177	414
5	28	133	99	533
6	29	162	78	611
25 c.c. N/1 NaOH added.				
7	33	195	36	647
5 c.c. N/1 NaOH added.				
8	32	227	41	688
9	20	247	44	732

The experiment showed that the bacteria can actively decompose some constituent of alley-bark with evolution of CO_2 and that it is practically impossible to destroy the resistant cells (spores) by dry heat ranging from 186° to 205°.



Text-fig. 3. The Fermentation of Alley-bark. Daily yields of Carbon dioxide. Unbroken line = test, broken line = control.

Some stack-bark was dried at 130° and portions weighing 30 grams were put into two flasks. Fifty c.c. of water were added to the test and 50 c.c. of 1 % mercuric chloride in 1 % sodium chloride to the control. Both were steamed for an hour on three successive days and were then connected up in the thermostat at 60°. No attempt was made to sterilise the bark with dry heat, and it was not seeded, as it was considered that the spores would be alive. Later films and growths showed this to be the case. The following shows the amount of carbon dioxide given off daily:—

Evolution of Carbon Dioxide from Stack-bark.

Days.	Control. Milligrams of CO_2.		Test. Milligrams of CO_2.	
	Daily.	Total.	Daily.	Total.
1	26	26	28	28
2	24	50	68	96
3	23	73	263	359
4	22	95	231	590
5	23	118	142	732
6	23	141	96	828
7	24	165	111	939
8	21	186	93	1032
added 10 c.c. water				
9	23	209	176	1208
added 10 c.c. water				
10	23	232	125	1333
11	22	254	75	1408
added 0·25 grams asparagin in 10 c.c. water				
12	48	302	166	1574
13	30	332	114	1688
14	31	363	113	1801
15	29	392	81	1882
16	25	417	62	1944

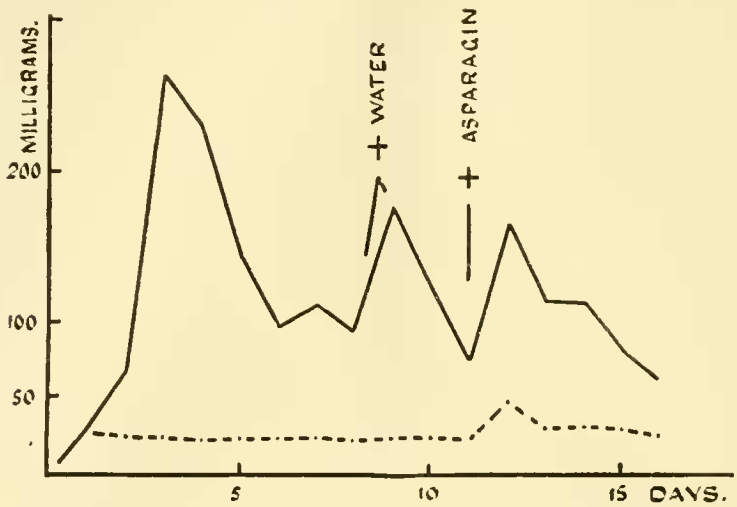
On the 8th day, the control was found to be sterile and the test to contain the stout rods with terminal spores. Although the neck and shoulders of the flasks showed water droplets, the barks themselves seemed to be rather dry and it was considered advisable to add some water. The idea was to add a solution of asparagin, but this was deferred on account of the appearance of the barks. They had become too dry, for 10 c.c. of water caused the yield of carbon dioxide to go up. By the 11th day the action had slowed down and 10 c.c. of water containing 0.25 gram of asparagin were added to each flask.

The asparagin increased the yield of carbon dioxide but not to the extent that the earlier experiment with tan-bark had led one to expect. But it was certainly curious that the asparagin should increase the gas in the control. This was unexpected and leads one to think that perhaps the control bark was not completely sterilised by the mercuric chloride added in the beginning. It is possible that some parts of the bark missed the action of the disinfectant and, if this is the reason, we must consider that the comparatively small yield of carbon dioxide by the control may be due, not to a chemical oxidation, but to a restrained bacterial fermentation. Tubes of saccharose media rubbed with fragments of the control bark gave no growths so that, as far as one can judge, the bark was sterile.

The experiment showed that 30 grams of dry stack-bark, when fermented with the native bacteria, gave off an excess of 1.25 grams of carbon dioxide in 11 days, and with the addition of 0.25 gram of asparagin a further excess of 0.37 gram in 5 days.

A fermentation experiment was made with viscose and Uschinsky's solution, but no carbon dioxide was given off during three days. A small quantity of

sodium hydrate was then added, but this had no effect. Upon investigating the matter it was found that Uschinsky's solution did not serve as a medium for growing the bacterium, and its inability to feed the organism could not be



Text-fig. 4. The Fermentation of Stack-bark. Daily yields of Carbon dioxide. Unbroken line = test, broken line = control.

traced to any one constituent. It had previously been found that a solution similar to Uschinsky's, but containing 1 % of dextrose and 0.3 % of potassium citrate, promoted the growth so that the presence of citrate or a soluble source of carbon is essential.

Meanwhile another fermentation test had been started with 25 c.c. of a modified Uschinsky's solution* and 4 grams of cotton wool that had been disintegrated by heating at 160° to 200°, until it could be rubbed into a fine powder.

No gas was given off in two days, so 5 c.c. of a 3 % solution of potassium citrate was added.

The citrate did not alter matters, for during the following two days, no gas was given off. This showed that in the earlier experiment, the growth and gas production depended, not on the citrate, but on the sugar.

So far the bacterium seemed incapable of attacking cellulose, but before closing the experiment it was thought advisable to see if, after a start had been made with extract of bark, the bacterium would attack the disintegrated cotton. Accordingly an extract was made by steaming 200 grams of alley-bark with 400 c.c. of water for an hour and filtering the extract, first through paper, then through porcelain. Ten c.c. of this were added to the test; the old control was thrown out and another prepared having everything the same as the test, excepting the 4 grams of cotton.

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* Asparagin 0.5 %, sodium chloride 0.3 %, magnesium sulphate cryst. 0.2 %, and monopotassium phosphate 0.2 %.

Experiment with Cellulose and Bark-extract.

Days.	No cellulose. Milligrams of CO_2.		Cellulose. Milligrams of CO_2.	
	Daily	Total.	Daily	Total.
1	0	0	0	0
	+ 1 c.c. N/10 NaOH.			
2	11	11	8	8
	+ 1 c.c. N/1 NaOH.			
3	1	12	1	9
4	1	13	31	40
5	0	13	16	56
6	0	13	7	63
7	0	13	5	68

One cannot say very much about this experiment for with 0.25 gram of asparagin and 4 grams of cellulose there should have been much more gas given off, and it would therefore appear as if the carbon dioxide had been derived from the bark extract. On the sixth day, the liquid control did not appear to have much bacterial growth although it contained active bacteria. The test flask contained a sodden mass of cotton with very little loose water. Both had the same slightly alkaline reaction to litmus and phenolphthalein papers. It is possible that if no alkali had been added on the second day the results might have been different.

Conclusions.

There are many points connected with the fermentation of the barks yet to be determined, but so far as the investigation has gone, certain facts have been elucidated. The active agent is a spore-bearing rod with an optimum temperature of 60° C. for laboratory work. It is capable of fermenting the spent wattle-bark of tanneries after the bark has been treated or "tempered." The tempering is undoubtedly a mixed fermentation and in it a temperature of from 40° to 50° is attained. Its object is to maintain a vigorous growth of the active bacteria, but incidentally a certain destruction of the organic matter by moulds, yeasts and bacteria probably occurs. Raw tan-bark which has been heated to a sterilising temperature is not easily fermented by the bacteria and it is probable that this is caused by the inhibiting action of the residual tannin products, because treatment of the heated raw bark with alkali and air so alters the bark that fermentation can ensue. Tests have yet to be made upon unheated raw bark, but it is likely that it will behave similarly to the heated bark. Heated tempered bark is easily fermented and is not influenced by treatment with alkali and air.

It will be remembered that when the stack is built, one part of new bark is mixed with four parts of tempered bark and when the stack is drawn the new bark is found to have been altered in character. It has become black and non-fibrous, indicating that some change has occurred and, as the bark is now easily fermentable, the assumption is that the long fermentation has, among other things, destroyed the inhibiting bodies, be they tannins or others.

In the stack there is a comparatively slow and prolonged evolution of carbon dioxide which is mainly, if not entirely, due to a bacteriological fermentation while the speed of the fermentation seems to be regulated by the small amount of nitrogenous matter in the bark. Were it to be mixed with some substance of a nitrogenous nature, there is no doubt that the production of carbon dioxide would be much more rapid. This was shown by the influence of asparagin in one or two experiments.

The organism is peculiar in some respects. It is capable of decomposing sugar such as dextrose or saccharose when freshly isolated, but soon after it requires the addition of alkali to enable it to act. There are some points yet to be determined regarding its viability, for the vegetative forms rapidly die off under certain, as yet uninvestigated, conditions. The spores are very difficult to destroy, especially when they are contained in the pores of the bark; they were alive after an exposure to 186° to 205° for two and a half hours.

While the bacteria can decompose tempered bark and stack-bark and also raw tan-bark after it has been treated with alkali, we are not quite sure if they are capable of fermenting cellulose such as disintegrated cotton-wool. When they were tested with this there was not enough carbon dioxide given off to warrant the conclusion that the cellulose had been attacked.

There was no growth of the organism in media devoid of soluble sources of carbon such as Uschinsky's solution. On the other hand there was growth and gas formation in similar solutions containing sugar. Thus the constituent of the bark that is fermented is still unknown.

Summary.

The fermentation of spent wattle-bark in the corrosion of white lead is caused by a stout rod-shaped bacterium having a terminal spore. Its optimum laboratory temperature is 60° C., although in the corroding stacks the temperature may rise to 80°.

Raw spent wattle-bark is difficult to ferment and requires a preliminary treatment. As conditions which oxidise tannin substances favour the fermentation of the raw bark, it is probable that the residual tannins inhibit fermentation.

I am indebted to Lewis Berger and Co., Ltd., for a supply of bark, and to Mr. H. J. Sullivan, of the Company, for notes upon the manufacturing process. I am also indebted to Mr. W. W. L'Estrange for much kindly assistance.